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CRUSTACEAN EYE-STALK HORMONE AND RETINAL PIGMENT MIGRATION

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INTRODUCTION

The study of color changes in crustaceans has for a long time occupied the efforts of investigators. Pouchet (1876) believed such changes to be under the control of the nervous system. But Perkins' (1928) and Koller's (1928) independent discoveries that a hormone, which effected concentration of the body pigment, was elaborated in the eye-stalks of *Palæmonetes* and of *Crangon* opened a fertile field for research. Since that time a series of studies by Koller and Meyer (1930), Smith (1930), Kropp and Perkins (1933), Hosoi (1934) and Hanström (1935) has shown this hormone to be present in a large number of crustaceans.

Brown (1935) has offered the opinion that more than one chromatophore-activating hormone is secreted from the eye-stalk into the blood stream. Hosoi's report (1934) that extracts of various organs of *Penæus japonicus* were able to concentrate the chromatophores of blinded *Paratya compressa*, might be taken to indicate that the chromatophore reaction is merely a non-specific reaction to protein extract. But I am inclined to attribute his results to traces of the hormone present in the blood coagulated within the various organs used.

This heretofore entirely physiological problem was given a morphological aspect by the discovery in crustacean eye-stalks of certain structures which seem to be secretory in character (Koller, 1930; Hanström, 1931, 1935; Sjögren, 1934). Brown (1933) and Hosoi (1934) reported the presence of the chromatophore activator in extracts of the ventral nerve cord, but no histological study of this organ has been made to discover similar secretory structures.

The complexity and similarity of this eye-stalk hormone to the pituitary secretions of vertebrates had already suggested itself to Kropp

(1932), who investigated the possibility of a gonadotropic effect on immature rats. In 1934 Navez and Kropp found that the crustacean chromatophore activator possessed growth-promoting properties when tested by means of decapitated coleoptiles of *Avena*.

Closely related to this subject of control of crustacean body pigments has been that of retinal pigment migration. The literature, up to within recent years, deals almost exclusively with such topics as the effects of light intensities, spectral colors, and temperature on the rate of migration. Only recently (Bennitt and Merrick, 1932) has there been any attempt to approach this problem from a chemical basis.

Parker (1897), in his study of the structure and positional changes of the retinal pigment cells in *Palæmonetes*, could find no nerves supplying the distal pigment cells. Still, the problem of interrelationship of the eyes, a controversial and as yet unsettled one in vertebrate physiology, was brought into the discussion. The crux of the problem, briefly, is this: that if retinal pigment migration is independent of the central nervous system, then the condition of the pigment in the light-adapted eye of an animal should have no effect on that of the other covered eye of the same animal, and, conversely, if the central nervous system is involved in retinal pigment migration, one eye may be "sympathetically" influenced by the condition of the other eye. The evidence presented on this point has been inconclusive. Parker (1897) believed from his experiments with *Palæmonetes* that retinal pigment migration was independent of the central nervous system, but von Frisch (1908) using *Palæmon* could get no decisive results. Castle (1927), who confined his study to the proximal pigment cells in the eyes of *Palæmonetes*, concluded that an illuminated eye was without "sympathetic" effect on the position of the pigment in the covered eye. Bennitt (1924, 1932a) worked with several decapod crustaceans and found that the condition of one eye influenced the other covered eye of the same animal.

In 1932 Parker suggested the possibility of a hormonal agent in retinal pigment migration, a factor already hinted at by Welsh (1930b) and by Bennitt. This field of research was suggested to me by Professor G. H. Parker, to whom I wish to express my thanks and obligations for many kindnesses. The specific problem undertaken was to investigate the action of the crustacean eye-stalk hormone on the retinal pigments.

MATERIAL AND METHODS

The extracts of the eye-stalks of the various animals used in these experiments were prepared in essentially the same manner. A number of stalks were excised at their proximal ends and were triturated by

means of a glass rod in a small Syracuse dish, the bottom of which had been previously roughened with powdered carborundum. The solvent was then added to this crushed tissue. Sea water was first used for this purpose, but was later abandoned in favor of amphibian Ringer's solution, since the latter was more constant in composition, being free from any dissolved foreign substances that might have occurred in the sea water, and could be used as a standard control substance.

After this preliminary treatment, the mixture was stirred for two minutes, as much of the coarse detritus separated off as possible, and the suspension of tissue in solvent drawn up into a hypodermic syringe. The extract was not filtered because of the small quantities prepared at one time. Control extracts of other tissues of the animals were prepared in the same way, or ordinary Ringer's solution with no tissue was used.

In those experiments where the extract was to be prepared from eyes adapted to darkness, use was made of two adjoining dark-rooms. The specimens were kept in one dark-room and were removed singly to the second room where, with the aid of a photographic safelight (Brownie Series No. 1) both eyes were excised and immediately crushed. With practice this operation could be performed within 30 seconds. It seems reasonable to assume that this short period of exposure to dim light would not induce in the eye the formation of a substance which might affect the retinal pigment. When a sufficient number of eye-stalks had thus been accumulated and crushed, the solvent was added and the process of extraction continued as above.

Injections of these extracts were made into the appropriate animals. In the first tests where extracts were injected into light-adapted shrimps little difficulty was encountered, but in later experiments the necessity of injecting in the dark-room into dark-adapted specimens presented certain technical difficulties. The initial results, disconcerting in their negativeness, were attributable to such factors as difficulty in capturing and removing an animal from the aquarium, penetration of the hypodermic needle too far into the shrimp with the result that the injected fluid entered the gill space, and injection of too large quantities of extract thereby causing the death of the specimen. With progress of the work, however, a satisfactory technique was evolved, which is considered of sufficient value to be described here in detail.

Before starting the experimental animals adapting to darkness, each shrimp was placed into a 600 cc. beaker containing about 300 cc. of sea water. The beakers were then transported to the dark-room and arranged in linear groups of five. Ten to twenty-five animals could thus be used conveniently for a single experiment. After an interval suffi-

cient to assure dark-adaptation the specimens were ready for injection.

Parker (1897) found that the time required for complete outward migration of the distal pigment cells in adaptation to darkness was from 105 to 120 minutes, while Welsh's (1930a) observations indicate that the time for this migration is only slightly less, from 90 to 120 minutes. In the early part of this study shrimps were left in the dark-room usually overnight, but this was later shortened to a period of 5 hours. This interval also suffices for complete dark-adaptation of the proximal and reflecting pigments. Adaptation to light was obtained by keeping specimens in a white porcelain dish which was illuminated from above by an electric lamp.

Upon entering the dark-room, the first animal in the series was removed from its beaker and held in the left hand. After orienting the specimen with its dorsal side uppermost, the tip of the hypodermic syringe, which was held in the right hand, was inserted between two of the abdominal tergites. As soon as the needle was felt entering, it was twisted downward and slipped forward a fraction of an inch, so as to enter the abdominal musculature obliquely. The plunger was then tapped gently downward about half a dozen times, the needle withdrawn, and the shrimp replaced in its beaker. Immediate exit was made from the dark-room to record the time and amount of extract injected. This process was then repeated for the entire series of animals in an experiment. With a little practice, manipulation of the hypodermic plunger could be well controlled, with the consequence that, in the several hundreds of specimens used in this study, amounts of extract ranging from 0.01 cc. to 0.05 cc. were injected. Occasional difficulty was encountered when an animal jerked while injection was being made. In some such cases the needle might emerge and part of the extract would be lost. All cases of this sort where there was any doubt as to whether the extract had entered or remained in the specimen were noted. It may be added, parenthetically, that there could be no absolute certainty that the complete volume of injected extract remained within the animal; a small part might have been expelled by the body movements of the shrimp, but it is hoped that this possible error was reduced to a minimum by injecting obliquely forward.

In order to make readings of the position of the pigment in the eyes of the experimental specimens, two methods were used. In one (Welsh, 1930a), where the experimental animal was adapted to light, observations were made chiefly on the living shrimp. To this end the bottom of a Petri dish was covered with a layer of soft paraffin from which a block about one-half inch square was cut, exposing the glass bottom of the dish. A shrimp was fastened down by means of sta-

tioner's staples so that its eye-stalks projected over this opening and the dish was then filled with sea water sufficiently to cover the specimen. By placing the vessel on the stage of a microscope, the position of the distal retinal pigment could be observed and recorded by means of a micrometer eye-piece. After an injection of eye-stalk extract, the animal could again be fastened down and the position of the pigment measured at suitable intervals.

In the second method where the specimens were adapted to darkness before injection of the stalk extract was made, it was necessary to fix the eyes before any readings could be taken of the position of the pigment. This was done by killing animals at varying intervals after injection by immersion in hot water (80°–90° C.) for 10 to 15 seconds. Following this preliminary fixation, the eye-stalks were excised, dehydrated, and cleared, either in oil of cedarwood or in beechwood creosote. The position of the distal pigment in the cleared eyes could then be measured. It was necessary to embed and section the eyes to observe the proximal and reflecting pigments. This was facilitated by first washing the cleared eye-stalks with ether-alcohol solution and then embedding doubly in celloidin and in paraffin. Sections were cut at 10 micra, some being subsequently stained with Delafield's hematoxylin and eosin, and others being mounted unstained.

The animals used in this study were all crustaceans of the order Decapoda. The common marine shrimp, *Palaeomonetes vulgaris*, was chosen as the test organism and, for a number of reasons, proved to be very favorable material. First, it is a commonly occurring form that is easily obtained in sufficient quantities. These specimens were readily secured in Boston from a dealer in live bait. During the summer months at Woods Hole they were caught by sweeping a dip-net along pilings and rocks covered with the alga, *Fucus*. Secondly, the mechanics and time periods for retinal pigment migration are well known through the excellent studies of Parker and of Welsh. Thirdly, the test shrimp, *Palaeomonetes*, does not experience the retinal condition of persisting diurnal rhythm (personal communication from Dr. Welsh) which has been found occurring in a number of crustaceans (Welsh, 1930b, 1935; Bennett, 1932b).

Animals whose eye-stalks were tested for the presence of a hormone that affected retinal pigment were: *Palaeomonetes vulgaris*, the common shrimp; *Uca pugilator*, the fiddler crab; *Libinia dubia*, the spider crab; *Cancer irroratus*, the rock crab; *Carcinides maenas*, the green crab; and *Callinectes sapidus*, the blue crab. The results of this investigation are considered below under separate headings.

STRUCTURE OF THE EYE OF *PALEMONETES*

For a detailed description of the finer structure of the eye and an account of the mechanics of pigment migration, the reader is referred to the papers of Parker (1891, 1897) and of Welsh (1930a, 1932). For this paper a description of the three types of pigment cells and their relation to the eye as a whole is deemed sufficient.

The eye of *Palæmonetes* is a compound structure consisting of ommatidial units. Composing or concerned with each ommatidium (Fig. 1) are the following structures: cornea (*C*), cone, rhabdome (*RH*), fenestrated basement membrane (*BM*), and the three types of pigment cells, the latter enriched by a varied terminology, but for purposes of simplicity and greater descriptiveness referred to here as distal (*DP*), proximal (*PP*), and reflecting (*RP*) pigment cells.

In each ommatidium there are two distal pigment cells which form a collar around the cone. These cells possess distal processes which extend to the cornea, and proximal processes which appear to be continuations of the proximal pigment cells.

There are eight proximal cells, one of which is rudimentary, to each ommatidium, all surrounding the rhabdome. Proximally beyond the rhabdome these cells become attenuated, pass through the fenestrated basement membrane, and, as retinal nerve fibers, connect with the first optic ganglion. These proximal cells appear to be the only retinal cells to have an anatomical connection with the central nervous system.

The reflecting pigment cells are located near the basement membrane. Processes from them may extend proximally through the basement membrane as far as the first optic ganglion, while distal processes may reach outward to the tip of the distal retinal cells where some of the reflecting pigment is accumulated in a small cap. The number of reflecting cells is difficult to determine unless sections are treated by special histological methods to remove the pigment and stain the nuclei, but Parker (1897) believes that there are no more than one or two of these cells for each ommatidium.

Black pigment granules are contained in both the distal and the proximal retinal cells. In the reflecting cells the pigment is of a different sort. When this pigment is viewed through the microscope by transmitted light, it appears yellowish or greenish brown and is barely distinguishable from the black pigment of the proximal and distal cells; examination of the same section by dark-field illumination, however, shows the reflecting pigment as a gleaming white granular mass. The pigment itself is probably guanine, though, as far as I am aware, few studies of the chemical nature of this white pigment have been made among the

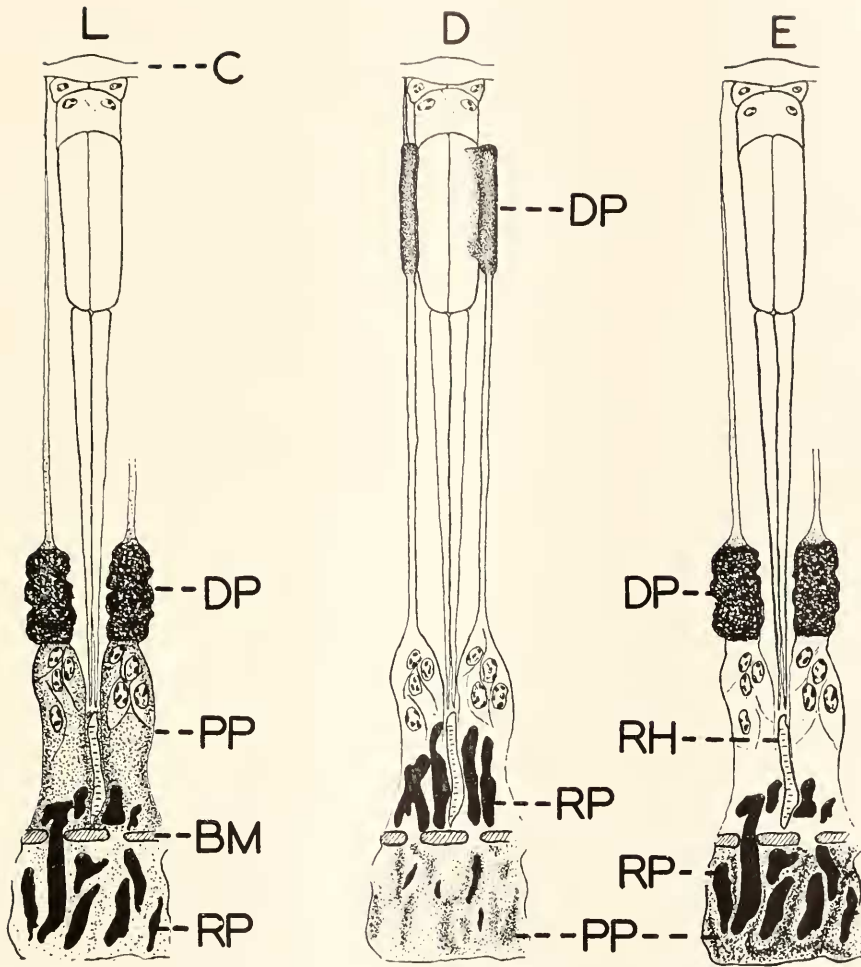


FIG. 1. Ommatidia from the eyes of *Palæmonetes vulgaris*, showing the general structure and the position of the three pigments under various conditions. *L*, from an eye in the light condition; *D*, from a dark-adapted eye; *E*, from an experimental animal which, after being adapted to darkness, was injected with stalk extract prepared from the eyes of light-adapted specimens. *C*, cornea; *DP*, distal pigment; *PP*, proximal pigment; *BM*, basement membrane; *RP*, reflecting pigment; *RH*, rhabdome.

invertebrates and none among the crustaceans. Guanin is commonly found occurring in crystalline condition in the integument of lower vertebrates (Ewald and Krukenberg, 1882, 1883). It is thought by some investigators to be formed from alimentary nucleoproteids and by others to be formed endogenously from nucleoproteids of the body tissues (Milot, 1922, 1923*a*, 1923*b*).

NORMAL PHOTOMECHANICAL CHANGES IN THE EYE OF PALEMONETES

The positions of the retinal pigment cells characteristic for the conditions in darkness and in light are shown in Fig. 1. The distal pigment cells in eyes which have become fully adapted to darkness have migrated distally so that they terminate approximately at the outward level of the cones. In this position, according to Exner (1891), lateral rays of light might be admitted through one cone and then be allowed to diffuse to the receptors of adjacent ommatidia, thereby increasing the efficiency of the eye.

In the light condition, the distal pigment cells have migrated proximally a distance of 150–200 micra, and have the form described by Herrick (1891) of a "plaited black ribbon." Parker (1897) regarded this appearance as abnormal, due to a prolonged stay in the dark before exposure to light, but Welsh (1930a) has found this to be a normal occurrence. I find this same condition to be typical in my preparations of light-adapted eyes. In this position the distal pigment cells prevent overstimulation of the rhabdome by excluding lateral rays and by admitting only those axial light-rays which enter the rhabdome by way of the cone.

The proximal pigment may be considered as moving in directions opposite to that taken by the distal pigment in response to light and to darkness. In the light, the pigment granules are found in a distal position above the basement membrane, while in a dark-adapted eye the pigment has migrated proximally below the basement membrane. The means by which the migration of the proximal pigment occurs has not been studied, but it has been assumed by Parker (1897), Mossler (1915), and Bennitt (1924) to result from some form of protoplasmic movement.

Exner (1891) first called the layer of reflecting pigment the tapetum. Its function appears to be the reflection of light from within the eye so that in darkness or in dim light, rays, which have entered the eye and have passed through the rhabdome, may be reflected back again into the rhabdome or even out of the eye. Stimulation may thus be accomplished by repeated application of weak light to the rhabdome, where ordinarily the light would have been of insufficient intensity to have any retinal effect. The glow which emanates from the eyes of many crustaceans which have been kept for some time in the dark is due to the reflection of light from the eye by the reflecting pigment.

It was believed for a time that the reflecting pigment did not change its position under various conditions of light, and from the common understanding that this pigment acted solely as a reflector no photomechanical change was considered necessary, so long as the proximal

pigment moved to cover or uncover the tapetum. But Parker (1897) thought that the reflecting pigment in *Palæmonetes* underwent positional changes in adaptation to light and to darkness. This was supported by reports of Trojan (1913) and of Mossler (1915) on *Palæmon*, and confirmed and extended by Welsh on *Palæmonetes* and on *Macrobrachium* (1932). In the dark-adapted eye the greater part of the reflecting pigment lies above the basement membrane (Figs. 1, 5, 9, and 10), while in a fully light-adapted retina much of it, except for a small portion, lies in processes below the basement membrane (Figs. 1, 6, 11, 12). Little is known about the mechanics of migration of this pigment.

Palæmonetes vulgaris

Four groups of tests involving the use of eye-stalk extract from this animal were carried out. In the first two groups, observations were made on living specimens in the manner already described above;

TABLE I

Proximal migration of the distal retinal pigment cells in a series of dark-adapted test *Palæmonetes* as a result of injecting individuals with 0.02 to 0.04 cc. of an extract of 60 eyes of light-adapted *Palæmonetes* in 1.0 cc. of sea water. Specimens were killed at various intervals after injection. The position of the pigment is given in ocular micrometer units (1 unit being equal to 16.2 micra) and was measured from the surface of the cornea to the distal margin of the pigment cells.

Minutes after injection	Each eye		Minutes after injection	Each eye	
0.....	4.0	3.5	25.....	13.0	11.0
0.....	3.0	3.5	28.....	14.0	17.0
0.....	5.0	4.5	30.....	15.0	15.0
5.....	5.0	6.0	32.....	17.0	16.0
10.....	6.0	6.0	34.....	13.0	12.0
15.....	9.0	6.5	36.....	19.0	19.0
18.....	12.0	10.5	38.....	16.0	16.5
20.....	8.0	12.0	45.....	15.0	20.0
21.....	11.0	11.5	50.....	19.0	18.0
22.....	15.0	17.0	55.....	18.0	19.5

in the last two, the eyes of the test shrimps were fixed, dehydrated, and cleared before measuring the migration of the distal retinal pigment.

In an attempt to determine whether the crustacean eye-stalk hormone would cause an elongation of the distal pigment cells in light-adapted shrimps, suitable specimens were injected with two kinds of extracts. Some received extract prepared from the stalks of shrimps that had been in the dark-room overnight, while others were treated with stalk extract from the eyes of animals which had been adapted to a black background. In neither of these cases was there any significant change in the position of the distal pigment cells (Kleinholz, 1934).

In the third group of tests, the entire procedure was reversed in order to see whether the eye-stalk hormone would induce a contraction

of the distal retinal pigment similar to that produced on the body pigment. Stalk extracts were prepared from the eyes of light-adapted specimens and were injected, in the dark, into test shrimps which had

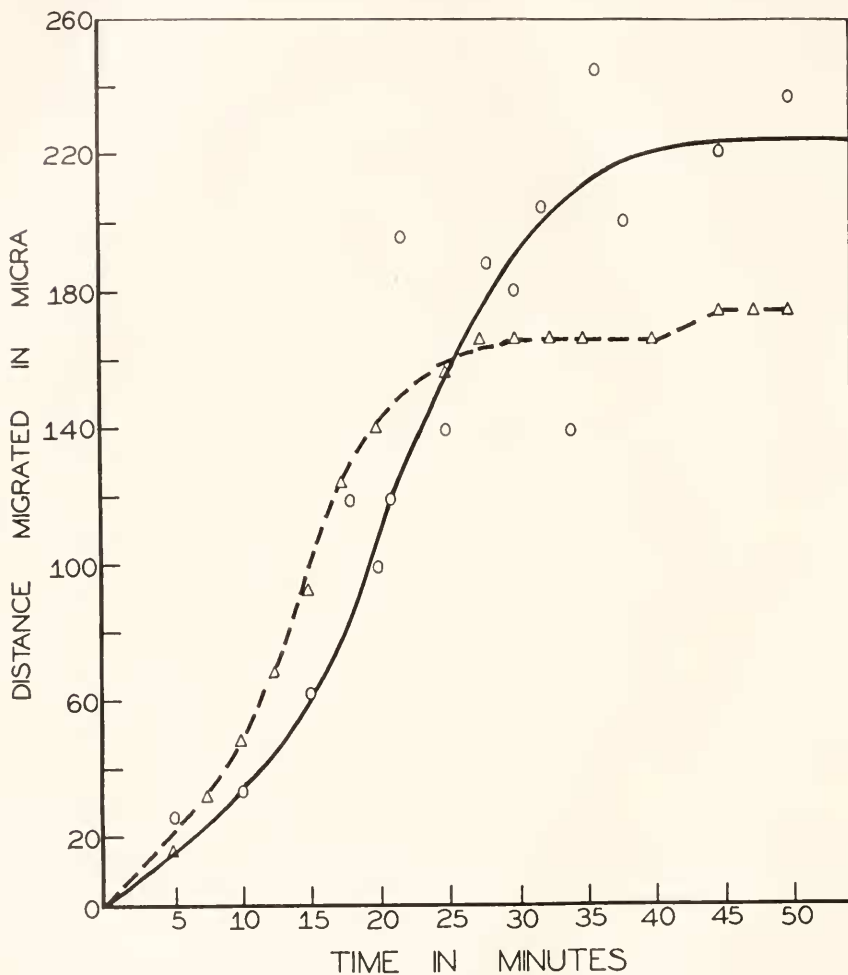


FIG. 2. Results of the measurements shown in Table I, plotted to show rate of migration of the distal pigment cells in a series of animals which have been injected, while adapted to darkness, with extract from the eye-stalks of light-adapted *Palaeomonetes*. The broken curve represents Welsh's figures on the rate of migration of the distal pigment during adaptation to light.

been adapting to darkness overnight. An extract of 60 stalks in 1.0 cc. of sea water was prepared and injected in doses ranging from 0.02–0.04 cc. into the animals of a series. After the preliminary fixing and clear-

ing of the stalks of the injected shrimps, the position of the distal retinal pigment was measured by means of a micrometer eye-piece. Proximal migration of this pigment had occurred, a maximum being reached in those animals killed from 30–45 minutes after injection.

The set of measurements for one such series is represented in Table I. The three uninjected controls were killed at intervals during the course of the experiment, one when injections into the test shrimps were about to be started, one at about the middle of the experimental period, and the third when the last injected specimen was fixed. Since the controls showed some variation in the position of the pigment, not only among themselves, but also between both eyes of the same animal, the average position of the distal pigment in all six eyes was taken as the "zero reading" for dark-adaptation.

The average measurements from both eyes of the test specimens of this series, when converted into micra and plotted against time after injection, give the curve (solid line) shown in Fig. 2. The broken-line curve in this figure is a transcription of Welsh's measurements on the rate of migration of the distal pigment cells during the course of adaptation to light. The general similarity of the two curves is at once evident. The rate of change is slow at first, but after 10 minutes is increasing rapidly, being 10 micra per minute for Welsh's curve, and about 6.5 micra per minute for mine. Welsh's figures show that the rate of migration begins to decline after about thirty minutes when the migration has neared its maximum, but that there is a secondary movement when the distal pigment cells contract into the plaited form observed by Herrick (1891). Since my measurements were made on a number of animals and are consequently subject to more variation than Welsh's consecutive readings on the same individual, I have not felt justified in indicating this secondary movement graphically, although from a study of my sectioned material it is known to occur.

The difference in rate of migration between the two curves invites interesting speculation. If we assume that in the dark no effective hormone is being released into the blood stream and that with light stimulation such a hormone is secreted until the pigment has contracted, we may expect that constant stimulation of the eye by light will result in a continuous secretion of the hormone to maintain the pigment in a state of contraction. Since my test specimens, however, were kept in the dark-room throughout the course of the experiment, the only hormone present within the body was that injected into the shrimps. The dosage and concentration used makes this equivalent to 1.5 light-adapted eye-stalks injected into each individual. The experimentally injected animals thus not only had a smaller amount of hormone than light-adapting

shrimps, but were also lacking a continued secretion which would occur during the process of adaptation to light. At this time, however, not very much importance can be attached to this aspect of the problem. More exact quantitative treatment must be delayed until the crustacean eye-stalk secretion can be isolated or prepared in purer form.

The scatter and the greater amount of migration obtained in my curve is due, I think, to variations in size of individual eyes and to the fact that my specimens were probably slightly larger than those used by Welsh.

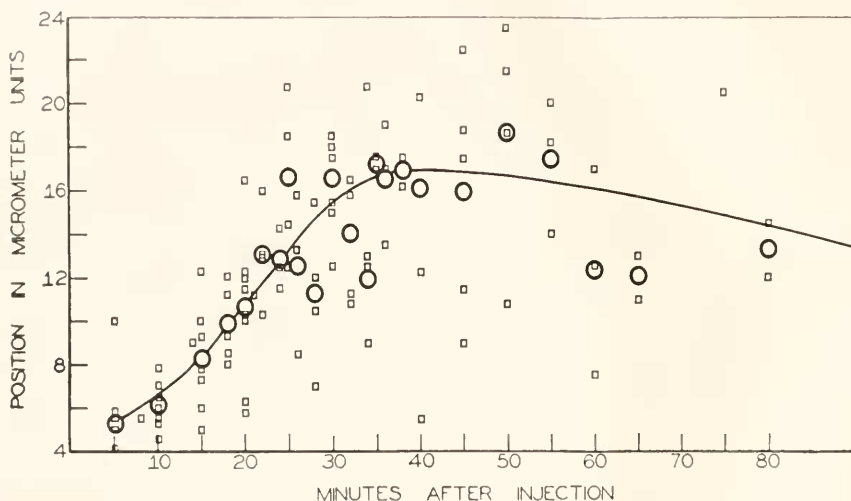


FIG. 3. Graph showing the change in position of the distal pigment in the eyes of dark-adapted *Palaeomonetes* at varying intervals after injection with light-adapted eye-stalk extract. Each square represents the average of the positions in both eyes of a single animal, and the larger circles represent the averages of all eyes measured at similar intervals after injection. The wide scatter shows the inadequacy of this method for quantitative measurements.

Any attempt at an exact quantitative determination of rate of migration of the distal pigment, made by killing individuals at varying intervals, is necessarily unsatisfactory. For rough estimates, these methods, used by Parker (1897), Mossler (1915), and Welsh (1930a), indicate the time required for complete adaptation to light or to darkness. But even so there is a considerable degree of deviation. Welsh reports, "In some eyes the distal pigment cells would have reached the extreme position characteristic for the light in thirty minutes, and in other eyes this would not be reached until sixty or seventy minutes in the light."

Mossler (1915) attempted to reduce this error by taking measurements on a number of eyes after each period of exposure to light and

then averaging the results. But this too was not very satisfactory. I have attempted a similar though more extensive method, using measurements made on about 200 eyes from dark-adapted *Palæmonetes* which had been injected with extracts of light-adapted stalks. The results are shown in Fig. 3. In this graph each square represents the average of the position of the distal pigment in both eyes of an experimental animal; the large circles represent the averages for all specimens killed at the same periods after injection. Except for about half a dozen specimens which showed little or no response to treatment with the eye-stalk extract, possibly because of inadequate injections, practically all of the other experimental animals showed some degree of distal pigment migration, although here too there is some discrepancy, even in animals killed at similar times after injection. These variations are reasonably attributable to such factors as individual differences in the size of the eyes and in the physiological conditions of the animals. Welsh (1930a) came to the conclusion that no really accurate measurement of the rate of migration of the distal pigment could be made except by studying this process in single individuals. Similarly, it is my opinion that any further quantitative studies of hormonal effects on this process will need to be made by a method identical with or similar to that finally used by Welsh.

When sections of eyes from experimental animals in this third group of tests were examined histologically, the proximal migration of the distal pigment was confirmed. The proximal pigment was apparently unaffected by the stalk extract, since it remained in the position typical for darkness. The reflecting pigment, however, was observed to have migrated proximally into the light condition. In an earlier paper, Welsh (1935) mentioned some of my unpublished results where the eye-stalk hormone was reported to have no effect on the reflecting pigment. At that time I had examined the first of my preparations only superficially and was misled by the difficulty of distinguishing, by transmitted light, between the pigment in the proximal and in the reflecting cells. Subsequent examination of the same and additional material by means of dark-field illumination showed that after injection of stalk extract the greater part of the reflecting pigment had moved below the basement membrane (Figs. 1, 7, 8, 13, 14).

Two control experiments were performed for this third group of tests. Whenever a series of *Palæmonetes* was put into the dark-room in preparation for injection with stalk extract, several primary control specimens were included along with it. These primary controls were uninjected and were fixed at intervals during the course of each series. The distal pigment in the eyes of such controls showed variations of

1-3 micrometer units (16-48 micra). Three series of secondary controls consisted of specimens which were first adapted to darkness and then injected either with sea water, cold-blooded Ringer's solution, or sea-water extract of shrimp's abdominal musculature. A total of 47 specimens were treated as secondary controls and in none of these was there any momentous change in the position of the distal pigment cells. The slight variation noted in the primary controls was also evident here, but this was undoubtedly due to differences in the eyes of individuals and has no significance for these experiments.

In the fourth group of tests, shrimps were adapted to darkness as before, but the stalk extract was prepared from *Palaeomonetes* which had been adapted to darkness. The method of preparing these dark-room extracts has been described above. Concentrations equivalent to 60 stalks in 1.0 cc. of amphibian Ringer's solution were injected in small quantities (0.02-0.04 cc.) into 41 dark-adapted test animals. The specimens were killed at an average time of 33 minutes after injection and the position of the distal pigment measured. A partial migration of the distal pigment towards the condition characteristic for light occurred, but this movement was not as marked as that resulting from the injection of extracts prepared from light-adapted stalks. For purposes of comparison the average position of the distal pigment in the eyes of this group are represented graphically with that of the third group where the same concentration of extract was injected into a nearly equal number of specimens (Fig. 4). Extracts from light-adapted eyes are seen to be almost twice as potent as those prepared from dark-adapted eyes. The probable significance of this difference will be discussed below.

Cancer irroratus

In examining the effects of stalk extracts from the eyes of various crustaceans on the retinal pigment of the test animal, *Palaeomonetes*, the chief interest centered on the response of the distal pigment cells which were most easily observable, but where possible, the fixed eyes of the test shrimps were sectioned to study also the reactions of the proximal and reflecting pigments.

Since it became apparent from the early work with *Palaeomonetes* extract that no reliable measurements could be obtained for the rate of response of the distal pigment to stalk extracts by the method used, it was decided to abandon this procedure, and, by a roughly quantitative method, attempt to study the effects on an "average" test specimen. Shrimps which survived treatment with a particular extract were killed at an interval of 30-40 minutes after injection. The average time the extract was allowed to act before killing the test animals and the average

TABLE II
Average time extract was allowed to act before killing animals and average position of distal pigment

Source of eyestalks	Number of animals injected	Number died	Average time killed after the injection <i>min.</i>	Number of eyes measured		Position of distal pigment		Average position of pigment	
				Injected	Control	Injected	Control	Injected	Control
<i>Cancer irroratus</i>	46	0	33.8	91	16	24,121	1,360	265.0	85.0
<i>Libinia dubia</i>	46	4	35.0	84	20	28,204	1,636	335.7	81.8
<i>Uca pugilator</i>	31	2	36.4	58	26	14,337	2,300	247.2	88.3
<i>Callinectes sapidus</i>	66	13	31.4	106	18	10,935	1,425	102.8	79.2
<i>Palamometes vulgaris</i> (light-adapted)	30	0	32.6	60	14	14,742	810	245.7	57.8
<i>Palamometes vulgaris</i> (dark-adapted)	41	6	32.5	70	18	10,465	1,409	149.5	78.3
<i>Carcinides manas</i>	34	6	38.0	56	19	11,485	1,506	205.1	79.3

position of the distal pigment for all the eyes studied in a series were then calculated and tabulated (Table II). The measurements obtained from the eyes of primary controls were treated in the same fashion. A number of secondary controls, consisting of test *Palae-monetes* into which various tissue extracts had been injected, were also studied, but these observations were not included in the tabulated results.

In this first series, 46 specimens of *Palae-monetes*, adapted to dark-

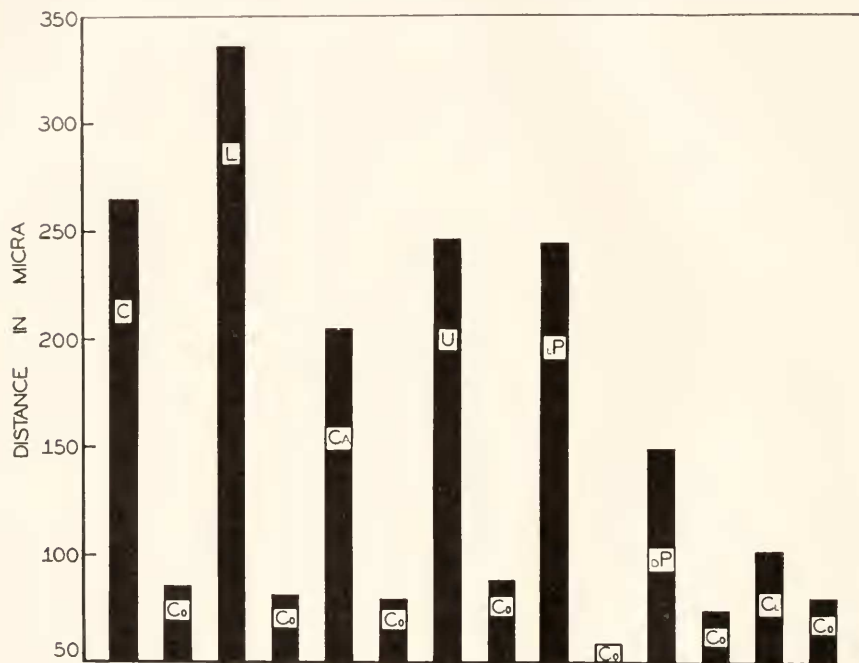
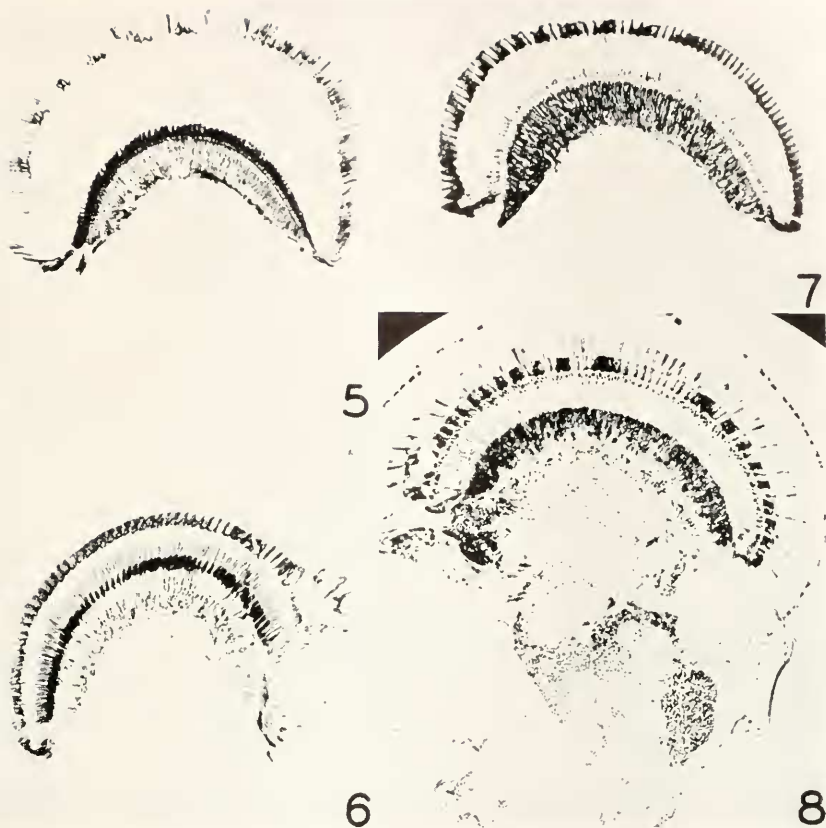


FIG. 4. Graphic representation of the position of the distal pigment in "average" test specimens as a result of injecting extracts from the eyes of various decapod crustaceans. Co, control uninjected specimens; C, extract from eye-stalks of *Cancer irroratus*; L, extract from eyes of *Libinia dubia*; CA, stalk extract from *Carcinides maenas*; U, extract from *Uca pugnator*; LP, extracts from eyes of light-adapted *Palae-monetes vulgaris*; DP, extracts from darkness-adapted eyes of *Palae-monetes vulgaris*; CL, extracts from eyes of *Callinectes sapidus*.

ness, were injected with small quantities (ranging from 0.02 to 0.04 cc.) of an extract of 10 light-adapted eye-stalks of *Cancer irroratus* in 0.8 cc. of amphibian Ringer's solution. The test animals were killed about 35 minutes after injection and the position of the distal pigment measured. The range of response was variable, as might have been expected from the earlier work. The lowest value (in one eye) was about 194 micra and the highest value (in one eye of another specimen) was



EXPLANATION OF PLATE I

FIG. 5. Longitudinal section of a normal retina of *Palaeomonetes vulgaris* in the "dark" condition. Only portions of the distal pigment cells show, due to the plane of sectioning. The reflecting pigment appears as a dense black mass above the basement membrane which is clearly visible. Below the basement membrane is the more dispersed proximal pigment. At the very bottom of the section are traces of reflecting pigment which apparently never migrates completely above the basement membrane.

FIG. 6. Longitudinal section of a normal light-adapted retina of *Palaeomonetes*. The distal pigment has migrated proximally. Immediately below the distal pigment is the diffuse proximal pigment which has migrated above the basement membrane. Below the proximal pigment is the mass of reflecting pigment from which many processes are seen extending below the basement membrane.

FIG. 7. Longitudinal section of a dark-adapted *Palaeomonetes* retina. This specimen was injected, in the dark, with 0.02 cc. of an extract of 20 light-adapted *Palaeomonetes* eye-stalks in 0.4 cc. of sea water. The retina was fixed 45 minutes after injection. The distal pigment has migrated proximally. The proximal pigment has remained below the basement membrane, while nearly all of the reflecting pigment has moved proximally below the basement membrane.

FIG. 8. Longitudinal section of the eye-stalk of a dark-adapted *Palaeomonetes*. This specimen was injected, in the dark, with 0.03 cc. of extract of 4 light-adapted *Libinia dubia* eye-stalks in 0.4 cc. of amphibian Ringer's solution, and was killed 35 minutes after injection. Stained with Delafield's hematoxylin and eosin. The position of the three pigments is the same as in Fig. 7.

372 micra; both of these specimens were killed at the same interval after injection of almost equal amounts of the extract. The average positions of the distal pigment for the 46 experimental (265 micra) and the 16 primary control shrimps (85 micra) are shown graphically in Fig. 4. Six secondary controls were injected with 0.02–0.05 cc. of an extract of cheliped muscle in Ringer's solution. The average time specimens were killed after injection was 30.5 minutes and the average position of the distal pigment was 69.2 micra.

The eyes of two of the test animals were embedded and sectioned. The proximal pigment was seen to have remained in the position characteristic for darkness, below the basement membrane, but the reflecting pigment had, for the most part, moved below the basement membrane into a position typical for light-adapted eyes.

Libinia dubia

In this experiment 46 test *Palaeomonetes* were injected with 0.01 cc. to 0.04 cc. of an extract of light-adapted eye-stalks from *Libinia dubia*. The concentration used, equivalent to 10 stalks in 1.0 cc. of Ringer's solution, was well tolerated by the shrimps, only 4 of them dying during the course of the experiment. Of the 42 surviving animals, two failed to show any response of the distal pigment, but all others showed a very marked contraction of the distal cells, in several specimens the position being measured at 437 micra from the margin of the cornea. The average position for the 42 surviving *Palaeomonetes*, including the two which showed no response at all, was 335 micra, and that for the primary controls was 82 micra. Five secondary controls were injected with extract prepared from the maxillipeds of *Libinia*. The average time during which the extract was allowed to act was 32 minutes, and the average position of the distal pigment at the end of this time was 76 micra.

EXPLANATION OF PLATE II

These photographs were taken by means of a Zeiss cardioid condenser.

FIG. 9. The same section shown in Fig. 5. This print has been retouched to indicate the expanded position of the distal pigment cells, made evident by the small caps of reflecting pigment at the distal ends of these cells. The main mass of the reflecting pigment lies above the basement membrane.

FIG. 10. A high-power detail of a portion of Fig. 9, showing the pigment above the basement membrane and the small amount at the proximal limit of the retina.

FIG. 11. The same section as shown in Fig. 6. The contracted condition of the distal pigment cells is indicated by the caps of reflecting pigment on the cells.

FIG. 12. A portion of Fig. 11 in high-power view. The reflecting pigment is seen extending proximally in processes below the basement membrane.

FIG. 13. The same as the section in Fig. 7.

FIG. 14. An enlargement of a portion of Fig. 13, showing the large number of processes containing reflecting pigment, due to a proximal migration induced by injection of eye-stalk extract.

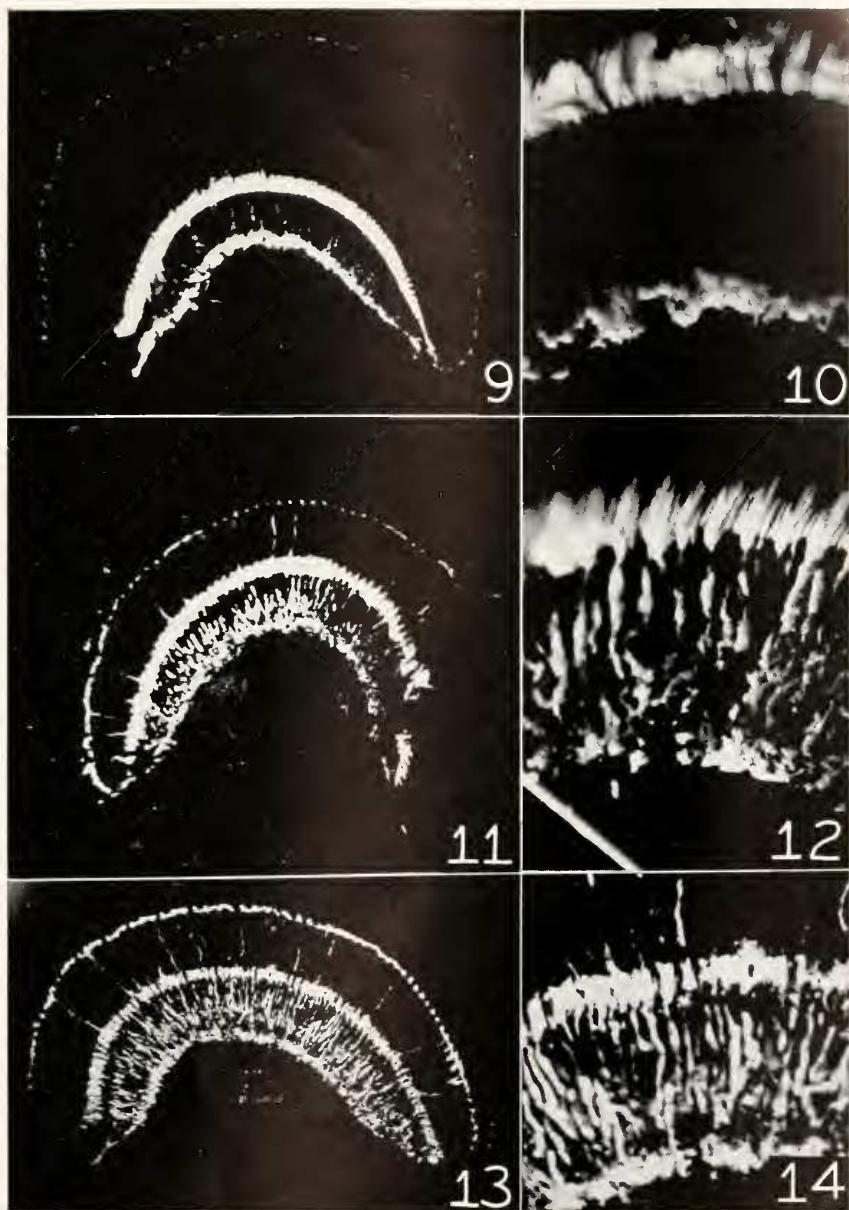


PLATE II

Sections of the eyes of three of the test *Palæmonetes* showed the proximal pigment persisting in the dark condition, but the reflecting pigment migrated into the light condition (Fig. 8).

Uca pugilator

Thirty-one test shrimps, which had been adapted to darkness, were injected with 0.02 cc. to 0.04 cc. of an extract of *Uca* eye-stalks, a concentration of 15 stalks to 1.0 cc. of Ringer's solution being used. Two of the test animals died during the course of the experiment, and only one of the 29 surviving *Palæmonetes* failed to show any change in position of the distal pigment; this exception was one that had been designated as doubtfully injected. In three eyes the position showing a small amount of migration was as low as 128 micra, but in the remaining eyes the range was up to a maximum of 356 micra. The average position for the test specimens was 247 micra, for the primary controls it was 88 micra, and for 5 secondary controls (cheliped muscle in Ringer's solution) the position was 68.0 micra.

The eyes of two of the *Palæmonetes* injected with extract from the stalks of *Uca* were sectioned. The proximal pigment was in the position characteristic for darkness, while the reflecting pigment showed a slight migration into the position characteristic for the light condition.

Carcinides maenas

In a preliminary experiment, 12 test *Palæmonetes* were injected with small amounts of *Carcinides* eye-stalk extract, in a concentration of 10 stalks per 1.0 cc. of saline solvent. This proved to be too concentrated, resulting in the death of 5 of the injected shrimps. With a slightly diluted extract (equivalent to a bit less than 8 stalks in 1.0 cc. of amphibian Ringer's solution) the survival was much better, only one of the test specimens succumbing to the injection. Five of the injected animals showed only very slight evidence of change in position of the distal pigment (the reading being at about 130 micra), while the remaining shrimps showed responses that were marked but slightly lower than those obtained previously with extracts from other crustaceans (Fig. 4).

The average position of the distal pigment in 26 surviving test specimens was 205 micra, that for 10 uninjected primary controls was 79.3 micra, and for 6 secondary controls injected with extract of cheliped muscle, the position was 76.5 micra.

No eyes were sectioned for observation of the proximal and reflecting pigments.

Callinectes sapidus

In early tests, the injection of extract prepared from the eye-stalks of *Callinectes*, in a concentration of 2 stalks to 1.0 cc. of Ringer's solution, produced a very slight, almost doubtful response of the distal pigment. In an attempt to increase the magnitude of the reaction, extracts of higher concentration (5 stalks to 1.0 cc. of solvent) were prepared and injected. Unfortunately, in these cases the crude extract proved to be very toxic, killing many of the test *Palæmonetes*. Those animals which did survive the injection did not show any increased response over those treated with the more dilute extract. The average position of the distal pigment for 53 surviving test specimens was 102.8 micra, and that for 9 primary controls was 79.2 micra. No secondary controls were carried for this series, and none of the eyes were sectioned for observation of the other retinal pigments.

The results obtained by using extracts prepared from eye-stalks of *Callinectes* are difficult to understand. Perkins and Kropp (1932) and Hanström (1935) found that stalk extracts prepared from this crustacean were effective in concentrating the body pigment of *Palæmonetes*; the potency of my extracts was tested by injection into dark *Palæmonetes* and the typical contraction of the body pigment was obtained. The response of the shrimp's retinal pigment to this extract is so slight as to be almost within the limit of experimental error. Two possibilities suggest themselves in explanation of this situation. There may be a difference in concentration threshold between the retinal pigment and the body pigment, and it may be that my extracts were not sufficiently purified (by filtering) to allow the use of more concentrated preparations which would not prove toxic. Or else, it is possible that crustaceans possess several pigmentary hormones, and that in *Callinectes* the hormone affecting the retinal pigment is lacking. This point requires further experimental investigation.

DISCUSSION

In studies of processes under hormonal control two general methods of procedure are open to the investigator. One is to remove the tissue thought to secrete the active agent, and the other is to inject extracts of the organs suspected of endocrine function.

Unfortunately, little use can as yet be made of the first method in studies of the crustacean pigmentary hormones, because there is a scarcity of information about the specific tissue within the eye-stalk which is involved in such activity. Only recently have there been any morphological studies of the crustacean eye that have revealed the pres-

ence of structures with a possible hormonal function. This has been reported by Hanström (1931, 1934) and one of his students, Sjögren (1934). These investigators found two different tissues, an X-organ and a "blood gland," which they think may play a part in the control of crustacean color changes. Professor Hanström has very kindly allowed me to read a preliminary paper of his which is in press at the time this paper is being written. He reports the findings of an experimental study made in an attempt to find a probable connection between the blood gland and the chromatophore activator. The results of a similar study on *Uca* have already been published (Carlson, 1935). According to Carlson, the middle third of the eye-stalk of *Uca* contains the active chromatophorotropic principle. Histological examination shows that the middle third of the stalk contains the well-developed blood gland, whereas the X-organ is either very small or completely absent. "In *Pagurus* . . . the blood gland . . . extends through both proximal and middle thirds of the eyestalk. The X-organ is situated in the proximal third, which thus contains part of the blood gland and the whole X-organ. Since the middle third of the eyestalk of *Pagurus*, that contains only part (the greater part, however) of the blood gland, concentrates the red and yellow pigment at least as strongly as the proximal third does, this fact, in connection with those mentioned above, speaks in favor of the view that the blood gland is the real source of the pigment concentrating substance in the decapod crustaceans" (Hanström, 1935). As Hanström recognizes, these facts do not exclude the X-organ from some part in the control of crustacean color changes. A more detailed knowledge of the rôles played by these two structures must await results from experimental removal or destruction of the endocrine tissues.

The second of these methods, used in this study, demonstrates fairly convincingly, that some endocrine organ within the eye-stalk is effective in causing two of the retinal pigments to migrate from a position that is characteristic for adaptation to darkness into a second position which is typical for an eye adapted to light.

It is interesting to note, in connection with this result, that a greater amount of migration of the distal pigment is obtained with the use of extracts prepared from eyes that were adapted to light, than with the use of extracts of an equal number of eyes which had been adapted to darkness. The explanation of this probably lies in the manner in which the eye-stalk hormone is synthesized and released by the animal. If the hormone is being formed continuously and stored until the time of release by light, acting as the stimulus, then there should be no difference in the potency of extracts prepared from light-adapted eyes and those

prepared from eyes adapted to darkness. If, on the other hand, no hormone is being built up in darkness, the response which was obtained from extracts of eyes adapted to the dark-room might be attributable to residual amounts of the effective hormone present in the gland through previous activity in response to light. This possibility merits cytological investigation.

In 1900, Gamble and Keeble reported a case of persistent rhythmic change in the chromatophores of *Hippolyte varians* even though the conditions of light remained constant. Slome and Hogben (1929) found a similar case of diurnal rhythm persisting in the South African frog, *Xenopus laevis*, after the removal of the normal stimuli. This animal, while kept in constant darkness, shows the melanophores more contracted at midnight than at midday.

Welsh (1930*b*) was the first to report a similar condition for the retinal pigment of crustaceans. Although *Macrobrachium olfersii* and *Macrobrachium acanthurus*, both nocturnal animals, were kept under constant illumination, the distal pigment continued to show a diurnal movement, migrating distally at 6:00 P.M. and remaining in this position until 5:00 A.M. the following morning, when it returned to the proximal position characteristic for light-adaptation. The proximal pigment did not exhibit such persisting rhythm.

Welsh's observations were soon followed by a similar one reported by Bennitt (1932*b*), who found that a rhythm persisted in the proximal pigment of the crayfish, *Cambarus*, even though the animal was kept in constant darkness. Subsequent studies made by Welsh (1935) found this phenomenon occurring in a number of other crustaceans. In *Latreutes fucorum* and in *Leander tenuicornis* the rhythm occurs in the reflecting pigment (and to a slight extent, perhaps, in the distal pigment) which, during the night, remains above the basement membrane in spite of constant illumination. *Leander affinis* shows "a definite rhythm in the movements of the reflecting pigment and a partial migration of the distal pigment" occurring "daily whether the animals are kept in constant light or darkness" (Welsh). *Penaeopsis goodii*, a distinctly nocturnal form, possesses a persistent rhythm of the proximal pigment, being similar to *Cambarus* in this respect.

The physiology of these activities in crustacean retinal pigment is as yet little understood. Welsh concluded from his experiments with *Macrobrachium* that the normal migrations of the distal pigment cells as well as their periodic movements under constant conditions of light were controlled directly by the blood and indirectly by the nervous system. The mechanism underlying this phenomenon of persisting rhythm has been too little studied to warrant any final conclusion. The fact that a

persistent rhythm may exist for one kind of pigment in one species, and for a second or third kind of pigment in another species, that it may persist in constant darkness for one crustacean and in constant light for another, all add to the complexity of the situation. Are we to attribute these different rhythms to several different hormones, or is there only one hormone to which these various pigment cells respond differentially because, let us say, of threshold differences? In *Macrobrachium*, is the rhythm due to liberation of a hormone, or is it due to cessation of hormone secretion? Is the nervous system directly involved in rhythms of the proximal pigment? If endocrine glands are involved, are they in the position of intermediate agents, secretion being started or stopped by a rhythm in the nervous system, or do the glands themselves function in a rhythmical fashion? All of these possibilities must be considered and tested experimentally before we can proceed to an understanding of this intensely interesting phenomenon.

SUMMARY

1. Specimens of *Palaeomonetes vulgaris* which had been exposed to light so that their retinal pigments were in positions characteristic for that state were injected with crustacean eye-stalk extract, in one series prepared from animals that had been in the dark-room overnight, and in a second series prepared from specimens that had been kept on a black background. There was no significant change in the position of the distal retinal pigment cells in either series.

2. When stalk extracts were prepared from light-adapted animals and were injected, in the dark, into *Palaeomonetes* in which the retinal pigments were adapted to darkness, a proximal migration of the distal retinal pigment occurred towards the position found typically in the eyes of specimens adapted to light.

3. If the rate of distal pigment migration following experimental injection is plotted, a sigmoid curve is obtained similar to that found by Welsh for the normal rate of migration when a single individual adapts to light.

4. Histological study of the eyes of experimentally injected *Palaeomonetes* shows that the reflecting pigment as well as the distal pigment has migrated into a position typical for a light-adapted eye. The proximal retinal pigment apparently undergoes no change in position.

5. Stalk extracts which were prepared from animals that were adapted to darkness, and which were injected into dark-adapted *Palaeomonetes*, in the dark-room, proved to be only half as potent as extracts prepared from the eyes of light-adapted shrimps.

6. Extracts of eye-stalks from *Cancer irroratus*, *Libinia dubia*, *Uca pugilator*, and *Carcinides maenas*, when injected into *Palæmonetes* which were adapted to darkness, also effected migrations of the distal and the reflecting retinal pigments. Stalk extracts from the eyes of *Callinectes sapidus*, in the concentrations used, had no effect on the retinal pigments of the test animals.

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